Supporting Information

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SI Materials and Methods

Cloning of Cyclin-Dependent Kinase 10 and Cyclin M cDNAs. We extracted RNA from the SH-SY5Y neuroblastoma cell line using an RNeasy kit (Qiagen). We performed RT-PCR reactions using a one-step RT-PCR kit (Qiagen) and the following oligonucleotide pairs. To amplify cyclin-dependent kinase (CDK)10 P1 cDNA: CDK10-5′: 5′-ATATGAATTCATGGCGGAGCCAGATCTGG-AG-3′; CDK10-3′: 5′-ATATCTCGAGTCAGGGTTTACAGCG-CTTGC-3′. To amplify CycM cDNA: CycM$_L$-5′: 5′-ATATGAA-TTCATGGAAGCCCCGGAGGGCGGC-3′; CycM-3′: 5′-ATA-TCTCGAGTTAGGGGATCTCTGTGTCCATGG-3′. The 5′ and 3′ oligonucleotides contain an EcoRI and a XhoI site, respectively. We purified the amplified products (coding for CDK10 P1,3,4 and CycM, Δ220–39, Δ144–53) using a PCR purification kit (Qiagen), and we ligated them into EcoRI/XhoI-cut pEG202 and pJG4-5 bait and prey yeast two-hybrid (Y2H) plasmids.

Plasmid Constructions. *Yeast two-hybrid plasmids.* The construction of CDK10 P1,3,4 and cyclin M full length, Δ220–39, Δ144–53 bait and prey plasmids is described above. We amplified CDK10 P2 cDNA (obtained from J.-Y. Thuret and F. Leteurtre, Service de Recherche en Hémato-Immunologie, DRM-DSV-CEA, Paris) using oligonucleotides CDK10-5′ and 5′-ATATCTCGAG TTAGCC-TGGCTCGCGGCACCCTTC-3′. We amplified the CycMΔexon5 and the CycMc201dupT coding sequences using the 5′ oligonucleotide CycM$_L$-5′ and the following 3′ oligonucleotides: 5′-ATATCTCGAG TTACTGCCACCACGGCTTCTCAGC-3′ and 5′-ATATCTCGAGTTAAAAGTACCTGT TGGACACATTG-3′, respectively. We amplified the N-terminal half coding sequence of ETS2 (v-ets erythroblastosis virus E26 oncogene homolog 2) (amino acids 1–172) using oligonucleotides 5′-ATATGAATT-CATGAATGATTTCGGAATCAAG-3′ and 5′-ATATCTCGA-GTTACT TTTCTTGGTTTTCTTTG-3′. We ligated the PCR products into EcoRI/XhoI-cut pEG202 and pJG4-5.

Mammalian cell expression plasmids. We amplified full-length CDK10 and cyclin M cDNAs from pEG202 plasmids using the following oligonucleotide pairs. For untagged CDK10: 5′-AGGCAAG-CTTACCATGGCGGAGCCAGATCTGGAGTGCG-3′ and 5′-AGCCCTCGAGTTATCAGGGTTTACAGCGCTTGCTCTG-GCC-3′; for V5-His tagged cyclin M: 5′-AGGCAAGCTTAC-CATGGAAGCCCCGGAGGGCGG-3′ and 5′-AGCCCTCG-AGGGGGGATCTCTGTGTCCATGG-3′. The 5′ oligonucleo-tides contain a *Hind*III site and introduce a Kozak consensus upstream of the ATG initiator. We ligated the PCR products into *Hind*III/XhoI-cut pcDNA 3.1/V5-His A (Invitrogen). We digested pEG202:CDK10-P1 with EcoRI and XhoI, and we ligated the insert into EcoRI/XhoI-cut pCMV-Tag 3B (Stratagene), a plasmid that directs the expression of Myc-tagged proteins. To express a CDK10 kinase-dead mutant, we performed site-directed mutagenesis on this plasmid using the QuikChange approach (Stratagene) and the oligonucleotide 5′-GAAGACAGCGAAT-TTCGGCCTG-3′ and its reverse complement.

We amplified the full-length ETS2 cDNA from the pCMV-SPORT6 (Imagene) plasmid using the oligonucleotides 5′-AT-ATCAATTGACCATGGATTACAAGGATGACGACGATA-AGGAATTCAATGATTTCGGAATCAAGAATATGG-3′ and 5′-ATATCTCGAGTCAGTCCTCCGTGTCGGGCTGGACG-3′. The 5′ oligonucleotide contains an MfeI site followed by a Kozak consensus and a Flag-tag coding sequence and an *Eco*RI site. The 3′ oligonucleotide contains an *Xho*I site. We ligated the *Mfe*I/*Xho*I digested PCR products into *Eco*RI/XhoI-cut pcDNA 3.1/V5-His A (Invitrogen). We performed site-directed muta-

genesis on this plasmid using the following oligonucleotides (and their cognate reverse complements): D-box mutant (RGTL->GGTV), 5′-GGCTAACAGTTACGGAGGGACAGTCAAG-CG CCAG-3′; S220A mutation: 5′-CTCCTGGACGCCATGT-GTCCG-3′; S225A mutation: 5′-GTGTCCGGCCGCCACAC-CCAGC-3′.

Insect cell expression plasmids. We digested pEG202:CDK10-P1 with EcoRI and XhoI and ligated the insert into EcoRI/XhoI-cut pGEX6P1 (GE Life Sciences). We amplified the GST-CDK10 coding sequence using the oligonucleotides 5′-GGCTCTAGA-ACCATGTCCCCTATACTAGGTTATTG-3′ and 5′-ATATG-CGGCCGCTCAGGGTTTACAGCGCTTGC-3′ that contain an XbaI and a NotI site, respectively. We ligated the PCR product into XbaI/NotI-cut pVL1393 (BD Biosciences). We digested the resulting plasmid with *Swa*I and NotI and cloned the insert into *Swa*I/NotI-cut pGTPb104a, a plasmid directing the expression of GST fusion proteins in the insect cell Bac-to-Bac system (Invitrogen). We amplified the full-length cyclin M cDNA from the pEG202 plasmid using the oligonucleotides 5′-ATATTCTAGA-ACCATGGCTAGCTGGAGCCACCCGCAGTTCGAAAAG-GCGCCATGGAAGCCCCGG AGGGCGGC-3′, which contains an XbaI site and the *Strep*-tag II coding sequence, and 5′-ATA-TGCGGCCGCTTAGGGGATCTCTGTG TCCATGG-3′, which contains a NotI site. We ligated the PCR product into XbaI/NotI-cut pVL1393. We digested the resulting plasmid with BamHI and NotI and cloned the insert into BamHI/NotI-cut pGTPb302, a plasmid directing the expression of protein in the Bac-to-Bac system.

Bacterial expression plasmids. We digested pcDNA3.1:Flag-ETS2 with EcoRI and XhoI and ligated the insert into M*fe*I/*Xho*I-cut pET15b (Novagen).

Tamoxifen Response Analysis. For the tamoxifen response analysis, we plated 12,000 cells per well into a 96-well plate, and we transfected them with SMARTpool or control siRNAs (Dharmacon) at a final concentration of 10 nM (20 nM for the combined transfection of CDK10 and cyclin M SMARTpools). After 24 h, we added various concentrations (10^{-9} to 10^{-6} M) of 4OH tamoxifen (Sigma Aldrich) and incubated for 4 d. We assessed cell viability using a CellTiter 96 Aqueous One Solution Cell Proliferation assay (Promega). Briefly, we removed the supernatant, added 3-(4,5-dimethythizol-2-yl)-5-(3-carboxymethox-yphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt solution (MTS) to the cells, incubated 1 h at 37 °C in the dark, and measured the absorbance at 490 nm. We measured four wells per condition.

Quantitative RT-PCR. We isolated total RNA from siRNA-treated MCF7 cells or lymphoblastoid cell lines using the RNeasy Plus kit (Qiagen). We generated cDNAs with the Reverse Transcriptase Core kit (Eurogentec) using the following gene-specific primers, picked from Primer Bank (http://pga.mgh.harvard.edu/primer-bank/). *CDK10*: forward 5′-GCCTGCGTCATCCGAACAT-3′, reverse 5′-AGGGTGTTGGCATATTCTCCAG-3′; *FAM58A*: forward 5′-AACCTGGACGCCTATGACC-3′, reverse 5′-GC-TCACCGCTTGGGTTAAAG-3′; *ETS2*: forward 5′-CCCCT-GTGGCTAACAGTTACA-3′, reverse 5′-AGGTAGCTTTT-AAGGCTTGACTC-3′; *c-RAF*: forward 5′-CCGAACAAGC-AAAGAACAGTG-3′, reverse 5′-GACGCAGCATCAGTA-TTCCAAT-3′; *GAPDH*: forward 5′-CTGGGCTACACTGA-GCACC-3′, reverse 5′-AAGTGGTCGTTGAGGGCAATG-3′.

We performed real-time quantitative PCR reactions on the cDNAs using the MESA Blue qPCR MasterMix Plus for SYBR

Assay (Eurogentec) and a LightCycler 480 System (Roche). We analyzed the results using the machine software, and we normalized all values with those obtained for GAPDH.

Mass Spectrometry. We excised from an SDS/PAGE 2 µg of recombinant 6xHis-ETS2 protein that we phosphorylated in vitro by CDK10/cyclin M. We corrected the pH with 100 mM NH_4HCO_3, removed the Coomassie blue with 50% acetonitrile/50% 50 mM NH_4HCO_3 (vol/vol), and dehydrated the gel with 100% acetonitrile. We reduced the protein in 10 mM DTT/100 mM NH_4HCO_3, and we alkylated it in 55 mM iodoacetamide/100 mM NH_4HCO_3. We briefly washed the gel with 100 mM NH_4HCO_3 and 50% acetonitrile/50% 50 mM NH_4HCO_3 (vol/vol), and we dehydrated it in 100% acetonitrile. We performed an overnight digestion with trypsin in 50 mM NH_4HCO_3. We extracted the resulting peptides using 50% acetonitrile/2% formic acid (vol/vol), and we loaded the samples onto an LC-MS/MS using a Triple TOF 5600 System (AB Sciex). We identified phosphopeptides using ProteinPilot (AB Sciex) and Mascot (Matrix Science) software. We localized the phosphorylated residues using Scaffold PTM (Proteome Software).

Production of Antibodies Against CDK10 and Cyclin M Recombinant Proteins. We produced 6His-CDK10 and 6His-CycM in bacteria [Origami 2(DE3) Singles Competent Cells, Novagen] 3 h at 37 °C, 0.2 mM isopropyl-β-D-1-thiogalactopyranoside. We lysed cells in

PBS supplemented with a protease inhibitor mixture (Roche) at 1.6 bars and centrifuged the cell lysate at $14,000 \times g$ 10 min 4 °C. We resuspended insoluble pelleted proteins with a 1% SDS solution. We performed an SDS/PAGE from which we excised approximately 200 µg of proteins in gel for first rabbit injections. We then produced GST-CDK10 and GST-CycM as described above (except for the temperature of 20 °C). We purified 150 µg of soluble GST-CDK10 and GST-CycM from the supernatant using a glutathione-agarose matrix following the manufacturer's instructions (Sigma). We injected these soluble proteins to boost the immune response. 67 d after the first injections, we collected rabbits' sera and purified total IgGs using a protein A column. These antibodies are now commercially available (Covalab).

Production of an Antibody Against a Cyclin M-Derived Peptide. We synthesized a 14-aa peptide corresponding to the carboxyl-terminal region of the mouse cyclin M ortholog. After purification we coupled it to hemocyanin and immunized a rabbit. Four weeks later, we performed immunogenic boosts every 3 wk for 9 wk, using the same antigen preparation. We collected the serum and performed an affinity purification using the initial immunogenic peptide. We tested the purified antibodies by Western blot analysis using cell lysates obtained from HEK 293 cells expressing cyclin M-V5-6His.
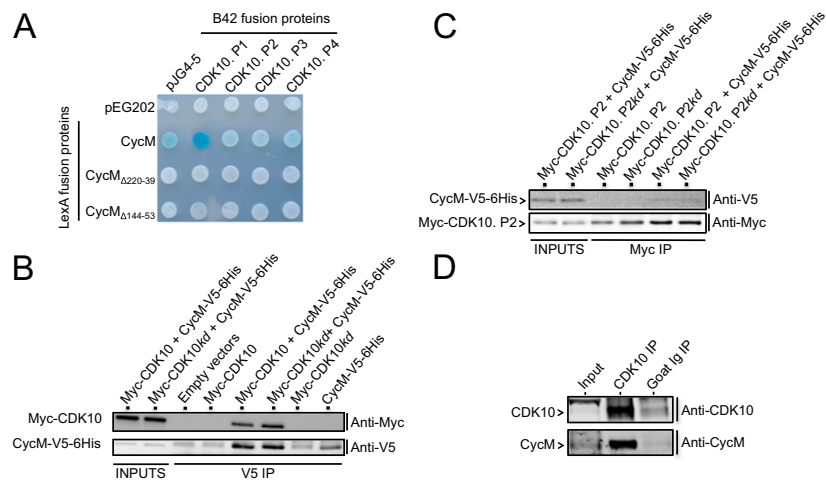
Fig. S1. Supplementary interaction data between CDK10 and cyclin M in yeast and human cells. (*A*) Y2H interaction mating assay between cyclin M (full-length and truncated isoforms corresponding to *FAM58A* splice variants) expressed as bait proteins (in fusion to LexA DNA binding domain) and the different isoforms of CDK10 expressed as prey proteins (in fusion to the B42 transcriptional activation domain). *lacZ* was used as a Y2H reporter gene. LexA-CycM shows a weak spontaneous activation of the reporter gene, but the Y2H phenotype is significantly stronger in the presence of the CDK10.P1 prey protein. (*B*) Western blot analysis of CycM-V5-6His immunoprecipitates obtained using the anti-V5 antibody, from HEK293 cells (co)transfected with different expression plasmids. "Inputs" correspond to 10 µg of total lysates obtained from HEK293 cells coexpressing Myc-CDK10(*wt* or *kd*) and CycM-V5-6His. (*C*) Western blot analysis of Myc-CDK10.P2 immunoprecipitates obtained using the anti-Myc antibody, from HEK293 cells (co)transfected with different expression plasmids. (*D*) Similar coimmunoprecipitation experiment to that shown in Fig. 1*F*, with a longer gel migration to discriminate between contaminating and CDK10 signal.

Fig. S2. Supplementary CDK10 silencing data. (*A*) Western blot analysis of Myc-CDK10 expression levels in MCF7 cells, in response to siRNA-mediated CDK10 gene silencing. (*B*) Western blot analysis of endogenous CDK10 expression levels in reponse to siRNA-mediated CDK10 or cyclin M gene silencing. A different, commercially available CDK10 antibody was used, compared with Fig. 3*A*.

Fig. S3. Supplementary data on the effect of CDK10 and/or cyclin M silencing on c-Raf protein levels, phospho-ERK protein levels, and tamoxifen sensitivity. (*A*) Quantification of c-Raf expression levels in response to CDK10 or cyclin M silencing, observed from the experiment shown in Fig. 3*B* and from two additional, independent experiments. *$P \leq 0.05$. (*B*) Western blot analysis of endogenous phospho-ERK1 and -2 expression levels in MCF7 cells, in response to siRNA-mediated silencing of CDK10 or cyclin M. (*C*) Cell viability assay of MCF7 cells transfected with 10 nM of control, CDK10, cyclin M, or CDK10 and cyclin M siRNAs and treated with different concentrations of 4OH-tamoxifen. ***$P \leq 0.001$, Student's test. Three independent experiments were performed for the individual SMARTpool transfections, and two independent experiments were performed for the combined SMARTpool transfection.
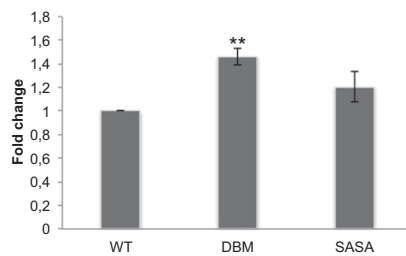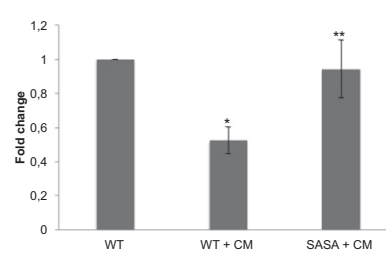
Fig. S4. Supplementary data on the expression levels of wild-type or mutant ETS2 proteins in response to silencing or overexpression of CDK10/cyclin M. (*A*) Western blot analysis of exogenously expressed Flag-ETS2 protein levels in MCF7 cells, in response to siRNA-mediated CDK10 or cyclin M silencing. (*B*) (*Upper*) Quantification of endogenous ETS2 expression levels in response to CDK10 or cyclin M silencing, observed from the experiment shown in Fig. 4*A* and from two additional, independent experiments. (*Lower*) Quantitative RT-PCR measurement of ETS2 mRNA levels in response to CDK10 (*Left*) or cyclin M (*Right*) silencing. *$P \leq 0.05$; **$P \leq 0.01$. (*C* and *D*) Quantification of the expression levels of Flag-ETS2 wild-type or mutant proteins in the absence of (*C*) or in response to (*D*) Myc-CDK10/cyclin M-6His-V5 coexpression, observed from the experiments shown in Fig. 4 *C* and *D* and from two additional, independent experiments. *$P \leq 0.05$; **$P \leq 0.01$.

MNDFGIK**NMDQVAPVANSYR**GTLKRQPAFDTFDGSLFAVFPSLNEEQTLQEVPTGLDSISHD
SANCELPLLTPCSK**AVMSQALKATFSGFKK**EQRRLGIPKNPWLWSEQQVCQWLLWATNEF**SL**
VNVNLQRFGMNGQMLCNLGKERFLELAPDFVGDILWEHLEQMIKENQEK**TEDQYEENSHLTS**
VPHWINSNTLGFGTEQAPYGMQTQNYPKGGLLDSMCPASTPSVLSSEQEFQMFPKSR**LSSVS**
VTYCSVSQDFPGSNLNLLTNNSGTPKDHDSPENGADSFESSDSLLQSW**NSQSSLLDVQRVPS**
FESFEDDCSQSLCLNKPTMSFKDYIQERSDPVEQGKPVIPAAVLAGFTGSGPIQLWQFLLEL
LSDK**SCQSFISWTGDGWEFKLADPDEVARR**WGKRKNKPKMNYEKLSRGLR**YYYDKNIIHK**TS
GK**RYVYR**FVCDLQNLLGFTPEELHAILGVQPDTED

Fig. S5. ETS2 peptide coverage of the mass spectrometry analysis. Residues labeled in bold, green characters have been covered by the mass spectrometry analysis. They represent 49.7% of the entire protein sequence. A total of 42 unique peptides were analyzed.

A

Conf. (%)	Peptide sequence	Modifications
99	GLLDSMCPASTPSVLSSEQEFQMFPK	Oxidations (M6, M23) – Carbamidomethylation (C7) – Phosphorylation (S17)
99	GLLDSMCPASTPSVLSSEQEFQMFPK	Phosphorylation (S5) – Carbamidomethylation (C7) – Oxidation (M23)
99	LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK	Phosphorylation (S10)
99	LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK	Phosphorylation (S5)
99	LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK	Phosphorylation (S5)
99	LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK	Phosphorylation (S5) – Deamidation (N25)
99	VPSFESFEDDCSQSLCLNKPTMSFK	Carbamidomethylation (C11) – Phosphorylation (S12)

B

Observed	Mr (expt)	Mr (calc)	ppm	M	Score	Expect	Rank	Peptide
994.7776	2981.3110	2981.2898	7.10	0	96	1.6e-009	1	GGLLDSMCPASTPSVLSSEQEFQMFPK
1000.1030	2997.2872	2997.2847	0.82	0	84	2.7e-008	1	GGLLDSMCPASTPSVLSSEQEFQMFPK
1103.8500	3308.5282	3308.5272	0.29	0	93	4.7e-009	1	LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK
1103.8580	3308.5522	3308.5272	7.55	0	65	4.3e-006	1	LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK
998.0893	2991.2461	2991.2378	2.78	0	46	4.5e-005	1	VPSFESFEDDCSQSLCLNKPTMSFK

C

Peptide	Position Ph-Ser	Manual confirmation
GGLLDSMCPASTPSVLSSEQEFQMFPK	220	Yes *(see Fig. S7)*
GGLLDSMCPASTPSVLSSEQEFQMFPK	225	Yes *(see Fig. S8)*
LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK	246 or248	Yes *(but ambiguity between two Serines; not shown)*
LSSVSVTYCSVSQDFPGSNLNLLTNNSGTPK	255	Yes *(not shown)*
VPSFESFEDDCSQSLCLNKPTMSFK	319	Yes *(not shown)*

Fig. S6. Identification of phospho-peptides and phospho-amino acids by mass spectrometry analysis. (*A*) Phosphopeptides identified by Protein Pilot with 99% or greater confidence. Putative localizations of posttranslational modifications are indicated. (*B*) Phosphopeptides identified by Mascot. Observed, *m/z* measured values; M_r (expt), theoretical mass of neutral peptide; M_r (calc), calculated mass of neutral peptide; ppm, parts per million; M, number of missed trypsin cleavage sites; Score, Ion Mascot score; Expect, statistical probability that the observed degree of ion matching would be found by chance. Rank, rank of the matches (1 indicates best matches). Putative localizations of modifications are indicated, with same color code as in *A*. (*C*) Summary of the phospho-amino acids confirmed by a manual analysis of spectra obtained from fragmented peptides, using Scaffold PTM.

Fig. S7. Identification of a phospho-serine at position 220. (*A*) Ion spectrum of fragmented peptide containing Ser220, generated by Scaffold PTM. (*B*) Zoomed-in view of spectrum corresponding to the orange-boxed region in *A*. Detection of a b8-98 ion (circled) indicates neutral loss of phosphate from ion b8 that corresponds to the GGLLDSMC sequence, in which Ser220 is the only potential phosphosite. (*C*) Spreadsheet representation of spectrum shown in *A*. Ions matching the spectrum are colored. Green boxes refer to neutral losses.

Fig. S8. Identification of a phospho-serine at position 225 and exclusion of a phospho-threonine at position 226. (*A*) Ion spectrum of fragmented peptide containing Ser225, generated by Scaffold PTM. Detection of b12-98 and b14-98 ions (both circled) indicates neutral loss of phosphate within the peptide GGLLDSMCPAST. Presence of b6 ion (without +80, nor neutral loss) indicates that Ser225 or Thr226 is phosphorylated. (*B*) Zoomed-in view of spectrum corresponding to the orange-boxed region in *A*. Detection of b11-98 ion (circled) indicates neutral loss of phosphate from ion b11 that corresponds to the GGLLDSMCPAS sequence, which does not contain Thr226. Hence, Ser225 is the only potential phosphosite. (*C*) Spreadsheet representation of spectrum shown in *A*. Ions matching the spectrum are colored. Green boxes refer to neutral losses.

Fig. S9. Confirmation of phosphorylation of serines 220 and 225 by CDK10/cyclin M in vitro. (*A*) In vitro CDK10/cyclin M kinase assay on ETS2 wild type and ETS2$_{SASA}$ recombinant proteins. ETS2$_{SASA}$ bears two alanine substitutions on Ser220 and -225. (*Upper*) Autoradiography of phosphorylated ETS2 proteins; (*Lower*) Coomassie staining of ETS2 added proteins. (*B*) Quantification of result shown in *A* and of results obtained from two other independent experiments. Autoradiography signals were normalized with Commassie signals. *$P \leq 0.05$.

Fig. S10. Supplementary data on the expression levels of ETS2 and cyclin M in lymphoblastoid cell lines derived from a STAR patient and from a healthy individual. (*A*) Quantification of endogenous ETS2 and cyclin M protein levels, observed from the experiment shown in Fig. 5*A* and from two additional, independent experiments. *$P \leq 0.05$; **$P \leq 0.01$. (*B*) Western blot analysis of endogenously and exogenously expressed cyclin M protein levels (same experiment as shown in Fig. 5*C* but using the anti-cyclin M antibody). (*C*) Quantification of endogenous ETS2 protein levels in response to cyclin M re-expression, observed from the experiment shown in Fig. 5*C* and from two additional, independent experiments. **$P \leq 0.01$.
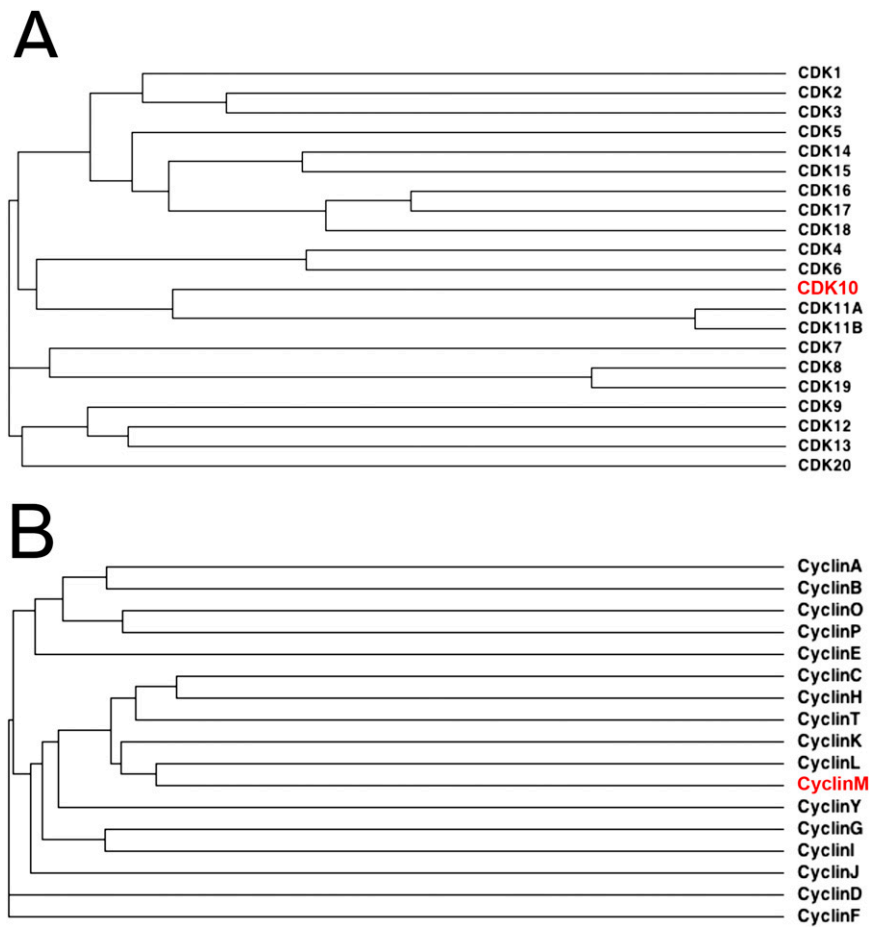
Fig. S11. Phylogenetic trees of human CDKs and cyclins. Protein sequences were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/protein/) and analyzed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). (*A*) CDK protein family. (*B*) Cyclin protein family.